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Prevention of Angiotensin II–Mediated Renal Oxidative Stress, Inflammation, and Fibrosis by Angiotensin-Converting Enzyme 2

JiuChang Zhong, Danny Guo, Christopher B. Chen, Wang Wang, Manfred Schuster, Hans Loibner, Josef M. Penninger, James W. Scholey, Zamaneh Kassiri, Gavin Y. Oudit

Abstract—Angiotensin-converting enzyme 2 (ACE2) is a monocarboxypeptidase capable of metabolizing angiotensin (Ang) II into Ang 1 to 7. We hypothesized that ACE2 is a negative regulator of Ang II signaling and its adverse effects on the kidneys. Ang II infusion ($1.5 \text{ mg/kg}^{-1}/\text{d}^{-1}$) for 4 days resulted in higher renal Ang II levels and increased nicotinamide adenine dinucleotide phosphate oxidase activity in ACE2 knockout (*Ace2*^{−/y}) mice compared to wild-type mice. Expression of proinflammatory cytokines, interleukin-1 β and chemokine (C-C motif) ligand 5, were increased in association with greater activation of extracellular-regulated kinase 1/2 and increase of protein kinase C- α levels. These changes were associated with increased expression of fibrosis-associated genes (α -smooth muscle actin, transforming growth factor- β , procollagen type I α 1) and increased protein levels of collagen I with histological evidence of increased tubulointerstitial fibrosis. Ang II-infused wild-type mice were then treated with recombinant human ACE2 ($2 \text{ mg/kg}^{-1}/\text{d}^{-1}$, intraperitoneal). Daily treatment with recombinant human ACE2 reduced Ang II-induced pressor response and normalized renal Ang II levels and oxidative stress. These changes were associated with a suppression of Ang II–mediated activation of extracellular-regulated kinase 1/2 and protein kinase C pathway and Ang II–mediated renal fibrosis and T-lymphocyte-mediated inflammation. We conclude that loss of ACE2 enhances renal Ang II levels and Ang II-induced renal oxidative stress, resulting in greater renal injury, whereas recombinant human ACE2 prevents Ang II-induced hypertension, renal oxidative stress, and tubulointerstitial fibrosis. ACE2 is an important negative regulator of Ang II-induced renal disease and enhancing ACE2 action may have therapeutic potential for patients with kidney disease. (*Hypertension*. 2011;57:314–322.) • **Online Data Supplement**

Key Words: angiotensin I ■ hypertension I ■ renal fibrosis ■ signal transduction

The ongoing epidemic of chronic kidney disease is a health care problem worldwide,^{1–3} and preventing the progression of chronic kidney disease to end-stage renal disease is one of the major goals of current nephrology practice. At the tissue level, kidney biopsy samples from patients with severe chronic kidney disease and end-stage renal disease exhibit prominent fibrosis, not only in the glomeruli and renal vasculature but also in the tubulointerstitium. Fibrosis in the tubulointerstitial compartment is the best histological predictor of clinical outcome in chronic kidney disease.^{4–8} Activation of the renin-angiotensin system and the subsequent generation of angiotensin (Ang) II (Ang II) is an important mediator of tubulointerstitial fibrosis and progression to end-stage renal disease.^{9,10} Angiotensin-converting enzyme 2 (ACE2) is a pleiotropic monocar-

boxypeptidase capable of metabolizing Ang II into Ang 1 to 7.^{11–14} In the cardiovascular system, ACE2 suppresses Ang II–mediated myocardial hypertrophy and fibrosis and prevents cardiac dysfunction.^{14,15} ACE2-deficient mice have age-dependent glomerulosclerosis develop¹⁶ and are more susceptible to diabetic renal injury,^{17,18} suggesting that ACE2 is a key modulator of kidney diseases. In this study, we directly assessed the hypothesis that ACE2 is a negative regulator of Ang II-induced renal injury and tubulointerstitial fibrosis.

Materials and Methods

Detailed methods are available in the online Supplement (available online at <http://hyper.ahajournals.org>).

Experimental Animals and Protocols

Mutant mice were back-crossed into a pure C57BL/6 background for >10 generations, as previously described.^{19,20} The 10-week-old male

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ACE2 knockout (KO) ($Ace2^{-/-}$) and their littermate wild-type (WT; $Ace2^{+/+}$) mice underwent in vivo Ang II infusion. An osmotic minipump (model 1002; Alza) was implanted subcutaneously at the dorsum of the neck to infuse Ang II ($1.5 \text{ mg/kg}^{-1}/\text{d}^{-1}$) or saline (vehicle) for 14 days. In a separate experiment, Ang II-infused WT mice were treated with placebo or recombinant human ACE2 (rhACE2; $2 \text{ mg/kg}^{-1}/\text{d}^{-1}$, intraperitoneal). All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996), Institutional Guidelines, and the Canadian Council on Animal Care.

Histology and Immunohistochemistry

Kidneys were fixed with 10% buffered formalin and embedded in paraffin. Picrosirius red staining and immunostaining for α -smooth muscle actin and CD3-positive lymphocytes were performed as previously described.^{21,22}

TaqMan Real-Time Polymerase Chain Reaction and Western Blot Analysis

RNA expression levels of various genes were determined by TaqMan (Applied Systems Inc, Streetsville, Ontario, Canada) real-time polymerase chain reaction as described previously.^{19,20,23} Expression analysis of the reported genes was performed by TaqMan reverse-transcription polymerase chain reaction using ABI 7900 sequence detection system; 18S rRNA was used as an endogenous control. The primers and probes for mRNA expression analysis by Taqman real-time polymerase chain reaction are listed in the online Supplemental Table I (available online at <http://hyper.ahajournals.org>). Western blot analysis was used to detect phosphorylated or total proteins or both as described previously.^{14,20,23}

Generation and Characterization of Human Recombinant ACE2

The extracellular domain of human ACE2 (amino acid residues 1–740; molecular weight = 101 kDa)²⁴ was expressed recombinantly in Chinese hamster ovary cells under serum-free conditions in a chemically defined medium as previously described.^{14,25} The enzymatic turnover of rhACE2 with Ang II substrate was $5.2 \pm 0.1 \mu\text{mol}/\text{mg}^{-1}/\text{min}^{-1}$. The purity of the expression product was 99.99% measured by high-performance liquid chromatography.

Ang Peptide Measurement

The excised kidneys were retrograde-perfused with cold phosphate-buffered saline solution to expel any remaining blood and quickly snap-frozen in liquid nitrogen. Renal Ang II and Ang 1 to 7 levels were measured by radioimmunoassay in the Hypertension and Vascular Disease Centre Core Laboratory at Wake Forest University School of Medicine as previously described.^{14,20}

Dihydroethidium Fluorescence

Dihydroethidium, an oxidative fluorescent dye, was used to measure superoxide (O_2^-) levels in kidney tissues from ACE2 KO and WT mice as previously described.^{14,20}

Lucigenin-Enhanced Chemiluminescence

The activities of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in kidney tissue were quantified by lucigenin-enhanced chemiluminescence as previously described.^{19,20}

Statistical Analysis

Results are shown as mean \pm SEM. Statistical analysis was performed using SPSS 11.5 software (SPSS) either by Student *t* test or by ANOVA, followed by multiple comparison testing (Student-Neuman-Keuls test) as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

Loss of ACE2 Worsens Ang II-Induced Tubulointerstitial Fibrosis, Renal NADPH Oxidase Activation, and Inflammation

Ang II (and its pro-oxidant effects) is a well-known activator of increased tissue fibrosis, including renal fibrosis.^{26–29} Loss of ACE2 enhanced the susceptibility to Ang II-induced tubulointerstitial fibrosis based on picrosirius red staining (Figure 1A). Activation of α -smooth muscle actin expression is a key marker of increased pathological fibrosis and immunohistochemical staining showed a mild elevation in WT kidneys, with a greater increase in ACE2 KO kidneys (Figure 1B) in response to Ang II. Expression analysis confirmed higher renal mRNA expression of α -smooth muscle actin (Figure 1C) and the fibrosis-associated genes, transforming growth factor- β (Figure 1D) and procollagen type I α 1 (Figure 1E), without a differential effect on procollagen type III α 1 expression (Figure 1F) in Ang II-infused ACE2 KO compared to WT mice. Western blot analysis confirmed increased protein levels of collagen I (Figure 1G), whereas collagen III (Figure 1H) levels were increased similarly in Ang II-treated ACE2 KO vs WT kidneys. Clearly, ACE2 deficiency increases Ang II-induced tubulointerstitial fibrosis.

In WT mice, infusion of Ang II resulted in a marked reduction in renal ACE2 protein levels (Supplemental Figure SIA, available online at <http://hyper.ahajournals.org>). We subjected ACE2 KO ($Ace2^{-/-}$) and WT ($Ace2^{+/+}$) mice to a 14-day period of Ang II infusion ($1.5 \text{ mg/kg}^{-1}/\text{d}^{-1}$). Renal Ang II levels were increased 4-fold in ACE2 KO kidneys compared with a 2-fold increase in WT kidneys without altering renal Ang 1–7 levels (Supplemental Figure SIB, C). Ang II-mediated NADPH oxidase activation and superoxide generation is a pivotal mechanism of Ang II-mediated renal injury.^{30–33} In the absence of ACE2, Ang II-induced superoxide production and NADPH oxidase activation were greater in ACE2 KO mice compared with WT mice (Supplemental Figure SID-F). Real-time polymerase chain reaction analysis of the expression of NADPH oxidase subunits revealed that loss of ACE2 augments Ang II-induced mRNA expression of p47^{phox} (Supplemental Figure SIG). Increased inflammation is a key mediator of Ang II-mediated injury,^{34–36} and as such we assessed for changes in expression of proinflammatory cytokines and chemokines. Expression of the cytokine interleukin (IL)-1 β and the chemokine chemokine (C-C motif) ligand 5 (CCL5) were significantly greater in ACE2 KO compared with WT kidneys, whereas the expression levels of tumor necrosis factor- α and IL-6 did not show a differential response (Supplemental Figure SIIA-D, available online at <http://hyper.ahajournals.org>). The increased renal Ang II levels, NADPH oxidase activation, and proinflammatory state resulted in greater phosphorylation of extracellular-regulated kinase (ERK) 1/2 pathways with increased protein kinase C (PKC)- α protein levels in the absence of a differential effect on PKC- β 1 levels (Supplemental Figure SIII-E-G). Together, these data show that loss of ACE2 increased renal oxidative stress, inflammation, and activation of pathological signaling pathways in response to Ang II.

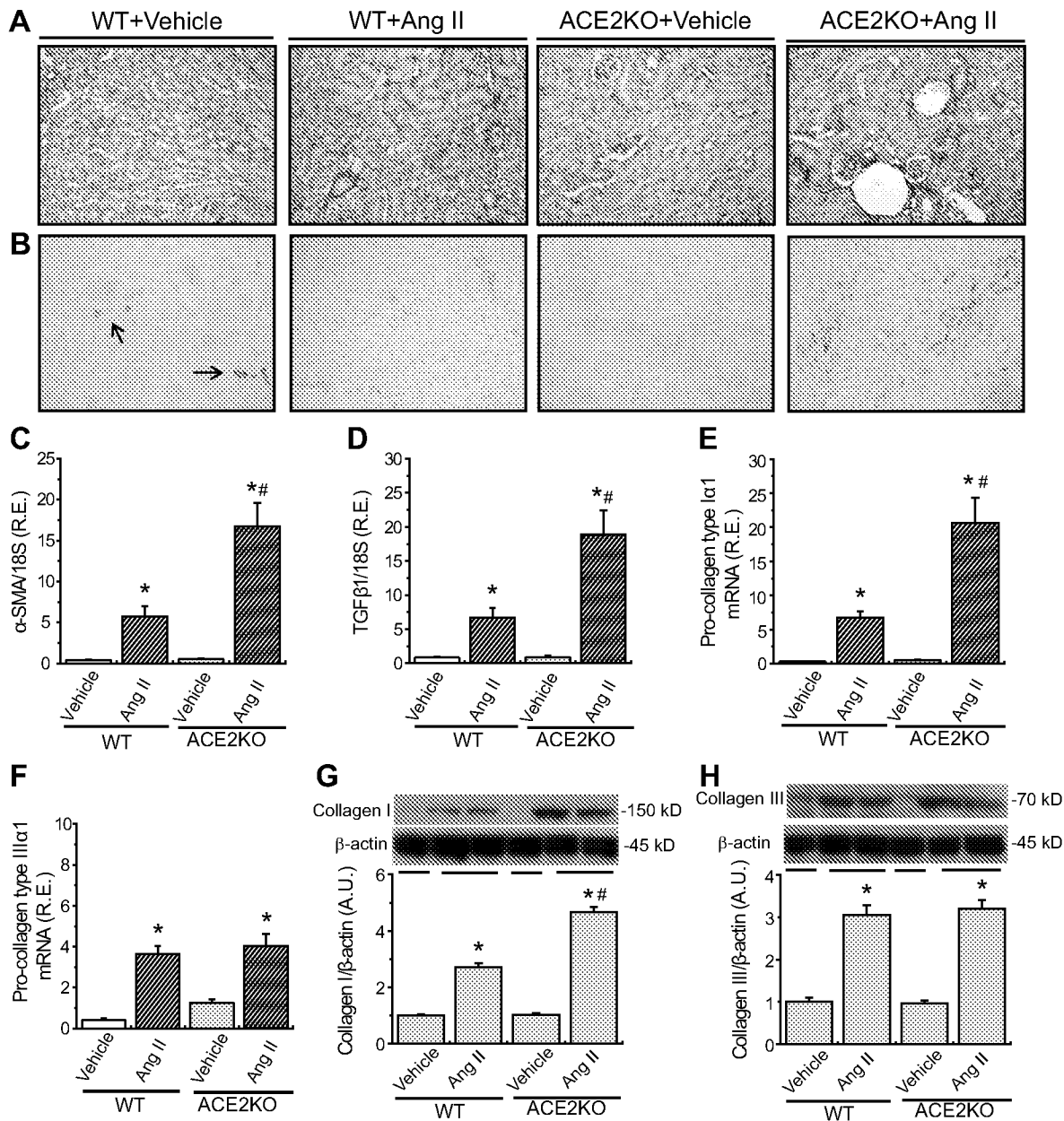


Figure 1. Angiotensin-converting enzyme (ACE) 2 is a negative regulator of angiotensin (Ang) II-induced tubulointerstitial fibrosis. Representative picrosirius red staining (A) and immunohistochemical staining of α -smooth muscle actin (SMA) (B) showing a greater increase in tubulointerstitial fibrosis and damage in response to Ang II in ACE2 knockout (KO) mice compared with wild-type (WT) mice. Arrows indicate positive control of α -smooth muscle actin in renal blood vessels. C–F, Taqman real-time polymerase chain reaction expression analysis showing greater elevation in the mRNA expression of α -SMA (C), transforming growth factor- β (G), and procollagen type I α 1 (E) without a differential effect on procollagen type III α 1 (F) in response to Ang II in ACE2 KO mice compared with WT mice ($n=6$ for WT+vehicle and ACE2 KO+vehicle; $n=8$ for WT+Ang II and ACE2 KO+Ang II). R.E., relative expression. Western blot analysis showing increased collagen I protein levels (G) without a differential effect on collagen III levels (H; $n=4$ for all groups). A.U., arbitrary unit. * $P<0.05$ compared with corresponding vehicle-treated group. # $P<0.05$ compared with WT+Ang II group.

Treatment With rhACE2 Reverses Ang II-Induced Renal NADPH Oxidase Activation and Proinflammatory Changes

We next evaluated whether rhACE2 treatment ($2 \text{ mg/kg}^{-1} \text{ d}^{-1}$, intraperitoneal) can prevent Ang II-mediated pathological remodeling in the kidneys. Two weeks of treatment with rhACE2 ($2 \text{ mg/kg}^{-1} \text{ d}^{-1}$, intraperitoneal) results in marked and persistent elevation in plasma ACE2 activity,¹⁴ with the development of extremely low levels of anti-

ACE2 IgG titers ($14.7 \pm 3.5 \text{ ng/mL}$) in only 7 of the 20 mice screened. Renal Ang II levels increased 2-fold in response to 2 weeks of Ang II infusion, which was normalized by rhACE2 (Figure 2A), whereas renal Ang 1-7 levels were unchanged (Figure 2B). In this model, plasma Ang II level is drastically elevated from a basal value of 38.5 ± 8.1 to $494 \pm 65.9 \text{ pg/mL}$ in response to Ang II infusion and is partially reduced by rhACE2 treatment ($212 \pm 51 \text{ pg/mL}$; $P<0.01$).¹⁴ Ang II infusion in WT mice

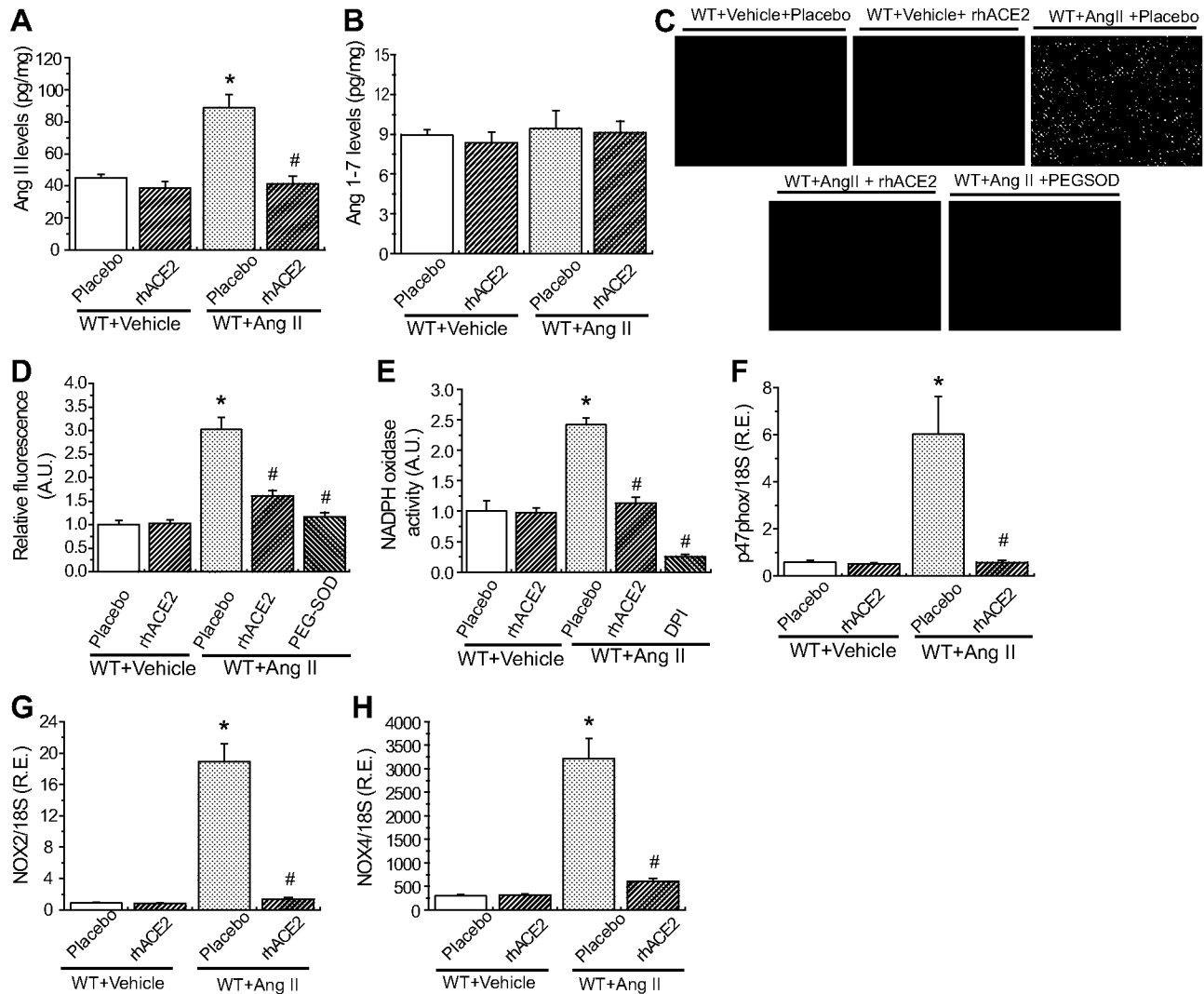


Figure 2. Treatment with recombinant human angiotensin-converting enzyme 2 (rhACE2) attenuated renal angiotensin (Ang) II levels, and Ang II mediated superoxide production and activation of NADPH oxidase activity. Renal Ang II levels completely normalized in response to rhACE2 in Ang II-treated WT mice (A) in the absence of a differential impact on renal Ang 1 to 7 levels (B) ($n=8$ for vehicle-treated groups; $n=14$ for Ang II-treated groups). Representative dihydroethidium fluorescence images (C), relative fluorescence values (D), and NADPH oxidase activity (E) showing a marked reduction of Ang II-induced superoxide generation and NADPH oxidase activity in response to rhACE2. NADPH, nicotinamide adenine dinucleotide phosphate; PEG-SOD, polyethylene glycol-superoxide dismutase (500 U/mL); DPI, diphenylene iodonium (10 μ mol/L). rhACE2 markedly attenuates the Ang II-induced expression of p47^{phox} (F), NOX2 (G), and NOX4 (H) subunits. R.E., relative expression. $n=6$ for placebo-treated groups. $n=8$ for rhACE2-treated groups. * $P<0.05$ compared with all other groups. # $P<0.05$ compared with WT+Ang II+placebo group.

for 2 weeks resulted in a significant increase in systolic blood pressure (168.2 ± 3.7 mm Hg; $n=12$; $P<0.01$), which was partially reduced with rhACE2 treatment (136.2 ± 3.3 mm Hg; $n=12$; $P<0.01$) compared to vehicle-infused WT mice receiving placebo (106.2 ± 3.1 mm Hg) or rhACE2 (104.2 ± 4.1 mm Hg; $n=8$). Ang II-induced increase in superoxide generation in the kidney was largely prevented by rhACE2 (Figure 2C, D) because of a suppression of the Ang II-induced activation of NADPH oxidase (Figure 2E). The superoxide scavengers, polyethylene glycol-superoxide dismutase (650 U/mL; Figure 2C, D) and diphenylene iodonium (10 μ mol/L; Figure 2E), were used to confirm the Ang II-mediated superoxide generation. Expression analysis confirmed a complete normalization of p47^{phox} (Figure 2F), NOX2 (Figure 2G),

and NOX4 (Figure 2H) mRNA expression in WT mice receiving Ang II and treated with rhACE2.

Consistent with a reduction in renal Ang II levels and superoxide production, expression of the inflammatory cytokines, including IL-1 β (Figure 3A), CCL5 (Figure 3B), tumor necrosis factor- α (Figure 3C), and IL-6 (Figure 3D), were restored to baseline values in mice infused with Ang II and treated with rhACE2. T-lymphocyte infiltration in the kidney markedly increased in response to Ang II, which was prevented by treatment with rhACE2 (Figure 3E; Supplemental Figure SIII, available online at <http://hyper.ahajournals.org>). Interestingly, rhACE2 completely normalized Ang II-induced increased phosphorylation of ERK1/2 (Figure 3F) and the increase in PKC- α (Figure 3G) and PKC- β 1 (Figure 3H) protein levels. These data illustrate the potency of rhACE2 to

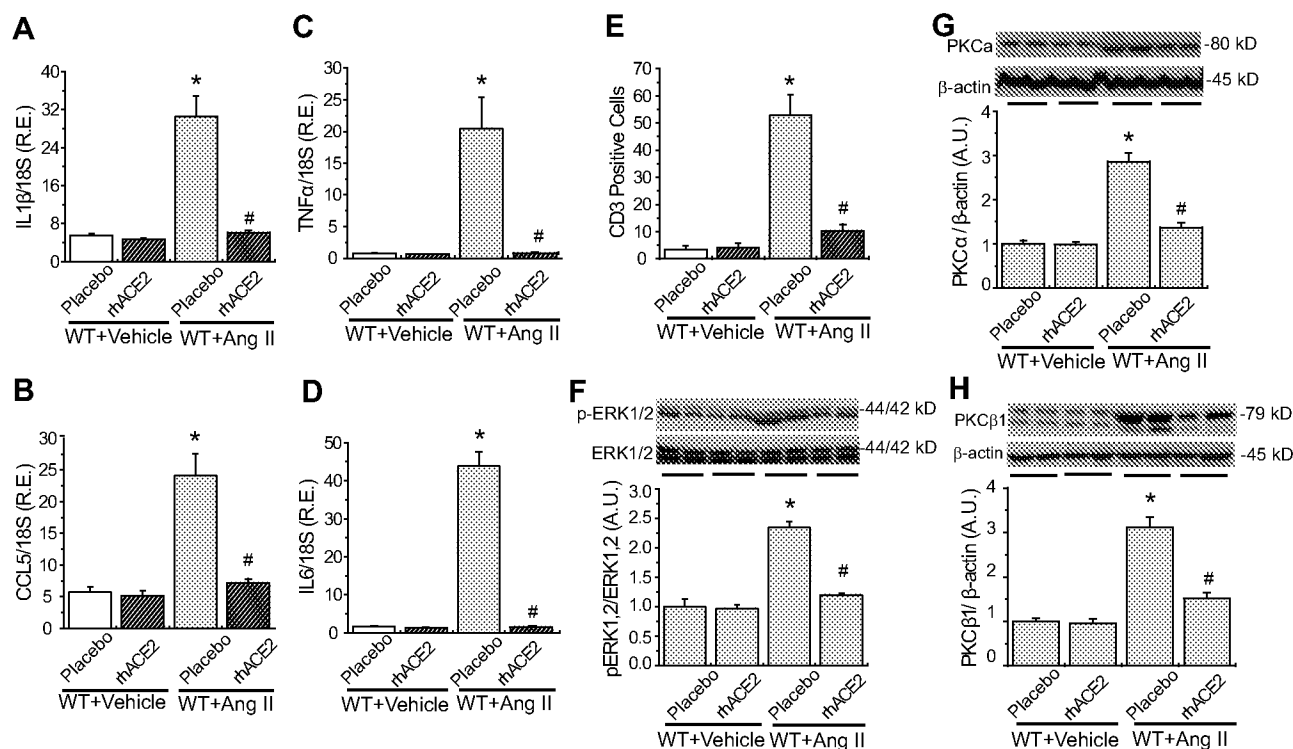


Figure 3. Recombinant human angiotensin-converting enzyme 2 (rhACE2) prevented angiotensin (Ang) II-induced proinflammatory state and upregulation of pathological signaling pathways. Taqman real-time polymerase chain reaction expression analysis of inflammatory cytokines showing normalization of Ang II-induced renal expression of interleukin (IL)-1 β (A), CCL5 (B), tumor necrosis factor- α (C), IL-6 (D), and renal T-lymphocyte infiltration based on CD3 immunostaining (E) by treatment with rhACE2. R.E., relative expression. $n=6$ for placebo-treated groups. $n=8$ for rhACE2-treated groups. Western blot analysis showing Ang II-induced phosphorylation of extracellular-regulated kinase (ERK) 1/2 (F) and increase in protein levels of PKC α (G) and PKC β 1 (H) was markedly suppressed by rhACE2. ERK1/2, extracellular-regulated kinase 1/2; PKC, protein kinase C. A.U., arbitrary unit; $n=4$ for each group. * $P<0.05$ compared with all other groups. # $P<0.05$ compared with WT+Ang II+ placebo group.

suppress Ang II-induced pathological remodeling and renal injury.

Treatment With rhACE2 Attenuates Ang II–Mediated Tubulointerstitial Fibrosis and Prevented Changes in ACE2 and AT1 Receptor Levels

Histological analysis confirmed that treatment with rhACE2 largely prevented the Ang II–mediated tubulointerstitial fibrosis, based on picrosirius red staining (Figure 4A) and immunostaining for α -smooth muscle actin (Figure 4B). The prevention of oxidative stress and the proinflammatory state suggest that rhACE2 also may have antifibrotic effects in the kidney. Treatment with rhACE2 reduced Ang II–induced mRNA expression of fibrosis-associated genes, including α -smooth muscle actin (Figure 4C), transforming growth factor- β (Figure 4D), procollagen type I α 1 (Figure 4E), and procollagen type III α 1 (Figure 4F) in WT mice. In agreement with these changes at the mRNA level, Western blot analysis of collagen I (Figure 4G) and collagen III (Figure 4H) showed a marked increase in response to Ang II, which was suppressed by rhACE2 treatment. Ang II–mediated renal fibrosis was also associated with decreased membrane-fractionated E-cadherin protein levels consistent with epithelial-to-mesenchymal transition, which was prevented by rhACE2 (Supplemental Figure SIV, available online at <http://hyper.ahajournals.org>). These findings clearly indicate that treatment with rhACE2 minimizes the adverse effects of Ang II on

the kidney, thereby preventing the development of tubulointerstitial fibrosis and damage. The complete normalization of renal Ang II levels and Ang II–mediated effects implies renal-specific changes were elicited by rhACE2. Western blot analysis revealed that rhACE2 prevented the Ang II–mediated loss of native ACE2 in the kidneys (Figure 5A) while normalizing the increased AT1 receptor levels (Figure 5B) without altering the level of the Mas receptor (Figure 5C).

Discussion

ACE2 is the first known homolog of human ACE and functions as a pleiotropic monocarboxypeptidase responsible for the degradation of a range of peptides with a high catalytic efficiency.^{11–13} We showed that loss of ACE2 promotes the progression of kidney disease by augmenting Ang II–induced renal injury. In contrast, rhACE2 prevented the molecular, signaling, and histological correlates of Ang II–mediated injury in the kidney. Ang II–mediated loss of renal ACE2 protein may serve as a positive feedback mechanism whereby Ang II–induced injury is perpetuated. In an ACE2-deficient state, Ang II infusion results in a greater pressor response,³⁷ whereas rhACE2 can suppress Ang II–induced pressor response in conscious mice.^{14,38} Ang II–induced pressor response is a key mediator of kidney damage,^{33,39} and in our experimental model the Ang II–induced pressor response was reduced by rhACE2.

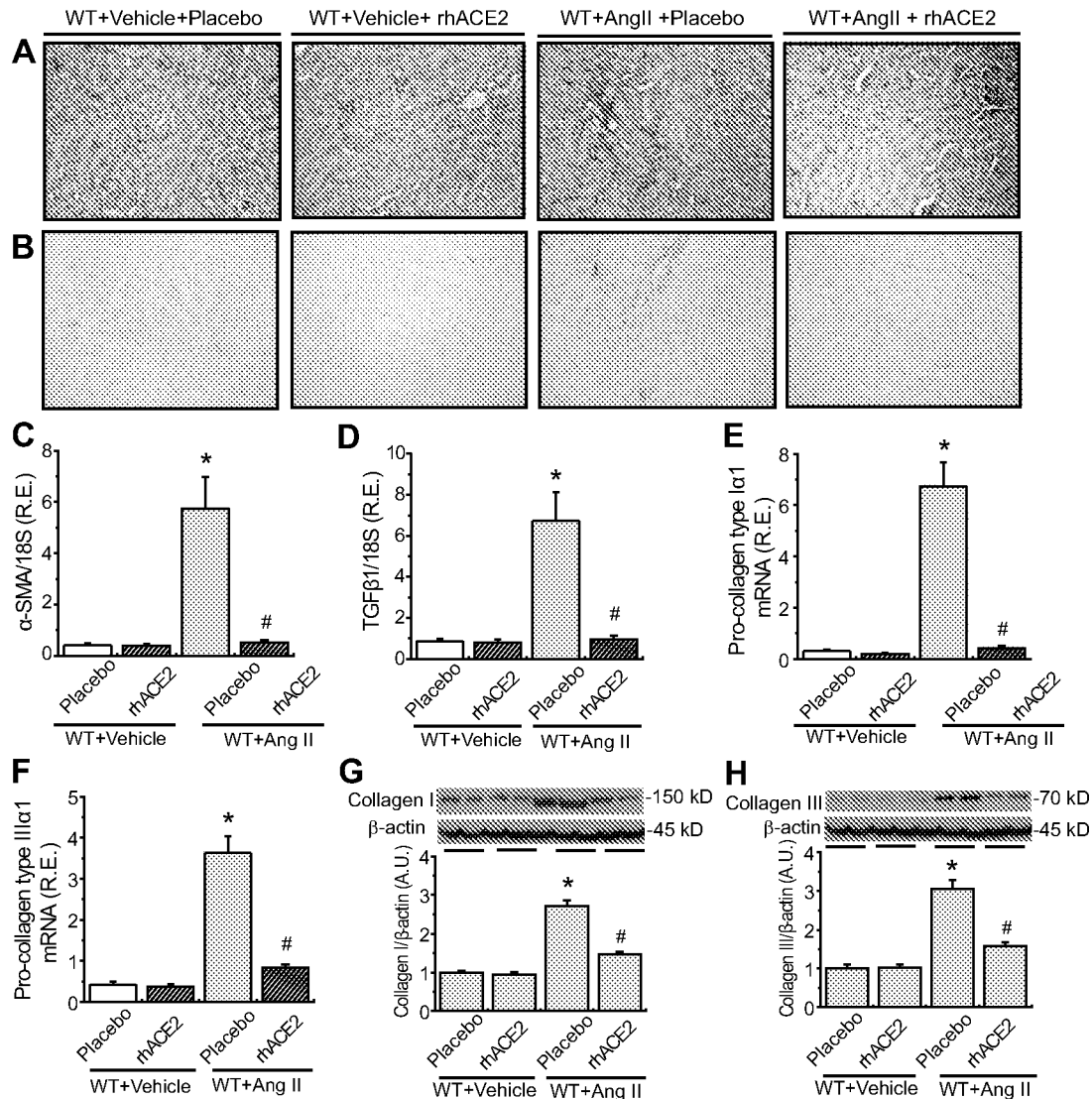


Figure 4. Treatment with recombinant human angiotensin-converting enzyme 2 (rhACE2) reduced angiotensin (Ang) II-induced tubulointerstitial fibrosis. Representative picosirius red staining (A) and immunohistochemical staining of α -smooth muscle actin (B) showing prevention of Ang II-induced increase in tubulointerstitial fibrosis and damage by rhACE2. Taqman real-time polymerase chain reaction expression analysis showing normalization of Ang II-induced mRNA expression of α -SMA (C), transforming growth factor- β (D), procollagen type I α 1 (E), and procollagen type III α 1 (F) in response to rhACE2. $n=6$ for placebo-treated groups. $n=8$ for rhACE2-treated groups. R.E., relative expression; α -SMA, α -smooth muscle actin; TGF β , transforming growth factor- β . Western blot analysis showing that treatment with rhACE2 prevented Ang II-mediated increase in collagen I (G) and collagen III (H) protein levels. $n=4$ for all groups. A.U., arbitrary unit. * $P<0.05$ compared with all other groups. # $P<0.05$ compared with WT+Ang II+ placebo group.

Regulation of renal Ang II levels, coupled with corresponding changes in plasma Ang II levels,^{14,37,38} highlights a key role of ACE2 in the metabolism of Ang II. In our experimental system, it is possible that the parallel infusion of ACE2 and Ang II may have contributed to inactivation of Ang II before absorption. ACE2 had a minimal effect on renal Ang 1 to 7 levels in our model. These results suggest that the potent and high-capacity ability of neprilysin^{40–42} and Ang II-activated ACE system to metabolize (and reduce) Ang 1 to 7^{43,44} are likely the major determinants of steady-state renal Ang 1 to 7 levels. The lack of a change in renal Ang 1 to 7 levels also may have resulted from AT1-mediated intracellular sequestration of Ang II, thereby rendering it resistant to Ang 1 to 7-producing enzymes. We linked changes in Ang II levels with variation in NADPH oxidase activation and O₂^{•-}

production. Renal NADPH oxidase, which is stimulated by Ang II, increases renal O₂^{•-} production and is linked to renal vasoconstriction, renal failure, and tubular dysfunction.^{45,46} Ang II activates a plethora of signaling cascades, including ERK1/2 and PKC signaling pathways.^{29,47,48} In this study, Ang II infusion resulted in elevated phosphorylation levels of ERK1/2 and increased protein levels of PKC- α and PKC- β 1 in WT mice, which were significantly enhanced by loss of ACE2 and suppressed by treatment with rhACE2, respectively.

Our data also show a clear role of Ang II-induced proinflammatory state and its ability to be modulated by ACE2. Ang II-mediated inflammation and T-lymphocyte infiltration has emerged as a key player in renal injury and hypertension.^{21,36,49} In particular, expression levels of IL-1 β and CCL5 were positively regulated in the absence of ACE2,

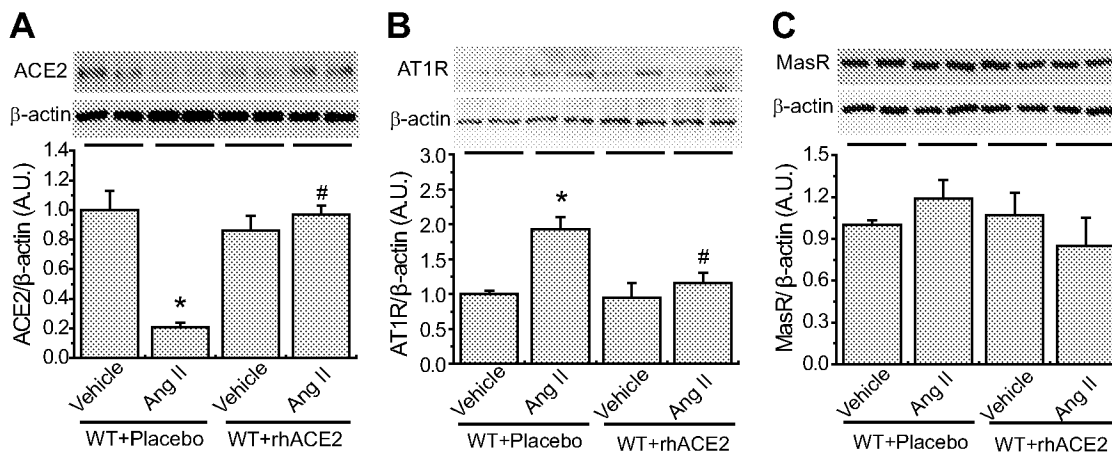


Figure 5. Treatment with recombinant human angiotensin-converting enzyme 2 (rhACE2) reduced angiotensin (Ang) II-induced decrease in ACE2 and increase in AT1 receptor levels. Western blot analysis and quantification showing that treatment with rhACE2 prevented Ang II-mediated decrease in renal ACE2 levels (A) and Ang II-mediated increase in renal AT1 receptor levels (B) without affecting the renal level of the Mas receptor (C). A.U., arbitrary unit. $n=4$ for each group. * $P<0.05$ compared with WT+Ang II+placebo group. # $P<0.05$ compared with WT+Placebo group.

whereas rhACE2 completely prevented their upregulation in response to Ang II. Consistent with previous findings,²¹ our results show that Ang II induces T-lymphocyte infiltration to the kidney, which is a known mediator of IL-1 β , IL-6, and the chemokine CCL5 production. Importantly, rhACE2 was able to completely suppress the infiltration of T lymphocytes. CCL5 is a member of the β -chemokine family that is chemotactic for monocytes/macrophages and lymphocytes, whereas IL-1 β activates T lymphocytes and plays a pivotal role in the regulation of inflammatory cell infiltration in a variety of renal diseases.^{50,51}

Ang II directly activates renal fibroblasts through its local tissue actions and renal fibroblasts express AT1 receptors, resulting in increased production of collagen and transforming growth factor- β .^{28,52} Loss of ACE2 augments Ang II-mediated mRNA expression of fibrosis-associated genes, including α -smooth muscle actin, transforming growth factor- β , and procollagen type I α 1, leading to increased tubulointerstitial fibrosis. Treatment with rhACE2 markedly decreased the expression of these fibrosis markers. Although treatment with rhACE2 resulted in a global normalization of expression pattern and signaling pathways, loss of ACE2 did not enhance Ang II-induced expression of tumor necrosis factor- α , IL-6, procollagen type III α 1, and PKC- β 1 levels. These differential responses suggest that Ang II infusion in WT mice reached a regulatory threshold level for the activation of these pathways such that the increase in renal Ang II levels in ACE2 KO mice resulted in no further increase. Recombinant human ACE2 also suppressed the Ang II-mediated increase in AT1 receptor and decrease in ACE2 protein levels, respectively, thereby eliciting renal-specific changes capable of further minimizing the detrimental effects of Ang II. The reduced AT1 receptor protein by rhACE2 may have decreased renal Ang II levels because of a reduction in the internalization of Ang II.

Inhibition of ACE2 function is associated with an age-dependent glomerulosclerosis,¹⁶ accelerated glomerular injury in Akita diabetic mice,¹⁷ and streptozocin-induced dia-

betes,^{18,53} providing definitive evidence that ACE2 is renoprotective and that reduced ACE2 expression might contribute to the progression of kidney disease.^{54–56} Moreover, rhACE2 slows the progression of diabetic nephropathy.²⁵ Taken together, these observations have given strong support to the concept that ACE2 plays an important role in negatively regulating renal injury. Enhancing ACE2 action by the use of rhACE2 or activator of endogenous ACE2⁵⁷ may minimize the progression of kidney disease.

Perspectives

In summary, loss of ACE2 enhances the susceptibility to Ang II-induced hypertension, renal oxidative stress, and pathological signaling, resulting in worsening tubulointerstitial fibrosis. In contrast, rhACE2 prevents Ang II-induced oxidative stress and pathological signaling. Ang II-induced pressor response is a key mediator of kidney damage and is controlled in part by ACE2. The exacerbation of Ang II-induced renal injury in ACE2 KO mice and the rescue by rhACE2 clearly is linked to an increase and decrease in renal Ang II levels, respectively. Given the upregulation of the renin-angiotensin system in kidney disease, loss of ACE2 function certainly can promote the progression of kidney disease by augmenting Ang II-induced renal injury. ACE2 may act as a protective mechanism in the kidney to limit the pathological activation of the systemic or local renin-angiotensin system or both.

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Disclosure

G.Y.O. is a Clinician Investigator Scholar of Alberta Innovates–Health Solutions and a Distinguished Clinician Scientist of the Heart and Stroke Foundation of Canada and Canadian Institutes of Health

Research. Z.K. is a New Investigator of the Heart and Stroke Foundation of Canada and a Scholar of Alberta Innovates-Health Solutions. M.S. is COO of Apeiron Biologics and owns stock in that company. H.L. is CEO of Apeiron Biologics and owns stocks in that company. J.M.P. is Founder of Apeiron Biologics and owns stock in that company.

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ONLINE SUPPLEMENT

Prevention of Angiotensin II-Mediated Renal Oxidative Stress, Inflammation and Fibrosis by Angiotensin Converting Enzyme 2

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Short Title: ACE2 Prevents Renal Oxidative Stress and Fibrosis

EXPANDED ONLINE METHODS

Experimental Animals and Protocols. Angiotensin converting enzyme 2 (ACE2) knockout mice ($Ace2^{-/-}$, ACE2KO) were backcrossed into the C57BL/6 background for at least 8 generations as previously described.¹⁻³ ACE2KO and their littermate wildtype ($Ace2^{+/+}$, WT) mice were used to undergo in vivo Ang II infusion. An osmotic minipump (model 1002; Alza, Palo Alto, Calif., USA) was implanted subcutaneously at the dorsum of the neck to infuse angiotensin (Ang) II ($1.5 \text{ mg.kg}^{-1}.\text{d}^{-1}$) or saline (Vehicle) for 14 days.^{3,4} Ang II-infused WT mice were then treated with placebo or recombinant human ACE2 (rhACE2; $2 \text{ mg.kg}^{-1}.\text{d}^{-1}$; i.p.) as described previously.^{3,5} Mice were housed in pathogen-free conditions and had access to sterilized food and water *ad libitum*. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), Institutional Guidelines and the Canadian Council on Animal Care.

Tail-Cuff Systolic Blood Pressure. For the measurement of tail-cuff systolic blood pressure (SBP), conscious mice were placed in the restrainers and their body temperature was maintained at $\sim 34^\circ\text{C}$ by the warming chamber. The IITC tail cuff sensor containing both the inflation cuff and the photoelectric sensor was placed on the tail and attached to the restrainer. The cuff was inflated to a pressure of 200 mmHg and then deflated slowly. Upon reappearance of pulse signals, SBP data from the IITC amplifier was recorded, analyzed and reported by the IITC software (IITC Life Science Blood Pressure System, Woodland Hills, CA). The mice were trained on three occasions before actual recordings were made and the corresponding SBPs were averaged from three readings and used for the averaged comparisons.

Histology. For kidney morphometry, kidneys were arrested with 1M KCl, perfuse-fixed with buffered 10% formalin, and embedded in paraffin. Trichrome and Picro-sirius red (PSR) staining and visualization were carried out as previously described.^{1, 2} Trichrome-stained sections were used for assessment of overall tissue architecture and interstitial and perivascular fibrosis. PSR staining of kidney sections were used to assess for tubulointerstitial fibrosis. Immunohistochemistry on paraffin embedded sections was carried out using a Ventana Discovery XT automated stainer (Ventana Medical Systems, Tucson, AZ) using the rabbit anti-human CD3 (clone Sp7; Lab Vision Corp., Fremont, CA) for T-lymphocytes. The anti-CD3 staining were performed in two kidney sections per experimental animal to obtain a mean score for each of them (n=4 animals per experimental group). The number of CD3-positive cells was randomly counted in a blinded manner from a total of 10 fields taken from both sections from each mouse.

Generation and Characterization of Human Recombinant ACE2. The extracellular domain of human ACE2 (amino acid residues 1-740, MW=101 kDa)⁷³ was expressed recombinantly in CHO cells under serum free conditions in a chemically defined medium. The expression product was purified to homogeneity by applying a capture step on a DEAE Sepharose® anion exchanger resin. The eluted fractions containing the expression product were submitted to a polishing step on a Superdex® 200 gel filtration column. The expression product was compared to the commercially available ACE2 standard 933-ZN (R&D Systems). Chemical and immunological properties of both products were almost identical while rhACE2 showed a 93% enzymatic activity with Mca-APK-(Dnp)-OH substrate in comparison to rhACE2 standard 933-ZN (R&D Systems). The enzymatic turnover of rhACE2 with Ang II substrate was $5.2 \pm 0.1 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ and the elimination half-life of rhACE2 was 10.4 hrs in Rhesus monkeys. The purity of the expression product was 99.99% measured by HPLC. Serum samples of mice were analyzed using an ACE2 antigen-specific enzyme-linked immunosorbent assay (ELISA) recognizing total anti-ACE2-specific IgG as previously described.⁴

ACE2 Genotyping Analysis. ACE2 gene mutant was confirmed by genotype analysis of ACE2^{-/-} vs. WT mice. For the amplification of ACE2 gene, specific primers were designed based on the GenBank data and synthesized by Sigma-Proligo. The sequence of the upstream primer was (5'-CCGGCTGCTCTTTGAGAGGACA-3'). The sequences of the down-stream primers were: (5'-CTTCATTGGCTCCGTTTCTTAGC-3') for wild-type genotyping and (5'-CCAGCTCATTCCTCCCACTC-3') for ACE2^{-/-} genotyping following the recommended protocol (Jackson Lab). A 25 μL polymerase chain reaction (PCR) reaction was performed which contained 3 μL genomic DNA, 2.5 μL 10x PCR Buffer, 0.5 μL each gene-specific primers, 0.2 mM dNTPs, 2 mM MgSO₄, and 0.25 unit of Taq DNA polymerase (Invitrogen). The PCR was performed for 30 cycles with temperature at 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 1 min on Mastercycler ep (Eppendorf AG). PCR products were assessed on a 1.2% agarose/Tris-Acetate-EDTA gel, stained with ethidium bromide and analyzed with the Image System.

TaqMan Real-time PCR. For various genes, RNA expression levels were determined by TaqMan Real-time PCR as previously described.^{1, 5, 9} Total RNA was extracted from flash-frozen kidney tissue using TRIzol reagent, and cDNA was synthesized from 1 μg RNA by using random hexamers. For each gene, a standard curve was generated using known concentrations of cDNA (0.625, 1.25, 2.5, 5, 10 and 20 μg) as a function of cycle threshold (CT). Expression analysis of the reported genes was performed by TaqMan Real-time PCR using ABI 7900 Sequence Detection System. The SDS2.2 software (integral to ABI7900 real-time machine) fits the CT values for the experimental samples and

generates values for cDNA levels. The primers and probes for mRNA expression analysis by Taqman Real-time PCR are listed in Supplementary Table 1 (see below). All samples were run in triplicates in 384 well plates. 18S rRNA was used as an endogenous control.

Lucigenin-Enhanced Chemiluminescence. The activities of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in kidney tissues of mice were quantified by lucigenin-enhanced chemiluminescence as previously described.^{2,5} The kidney homogenates (200 µg total proteins) were collected in 100 µl of phosphate buffer solution (PBS) mixture with protease inhibitor (Calbiochem, San Diego, USA) and phosphatase inhibitor cocktails (Sigma-Aldrich, Oakville, Canada) and then centrifuged at 1000 g for 10 min. The supernatants were then collected and added NADPH (1 mM) and lucigenin (50 µM) for NADPH oxidase activities assay with FB-12 luminometer in the presence or absence of diphenylene iodonium (DPI, 10 µM), a selective inhibitor of flavin-containing enzymes including NADPH oxidase. Data were calculated as the change in the rate of luminescence per minute per milligram of tissue.

Dihydroethidium Fluorescence. We used oxidative fluorescent dye dihydroethidium (DHE) to measure superoxide (O_2^-) levels in kidney tissues from ACE2KO and WT mice as previously described.^{2,4} For kidney samples, 20 µm fresh frozen tissue sections were washed with hanks balanced salt solution (HBSS) with magnesium and calcium and then incubated at 37 °C for 30 min with DHE (20 µM) in HBSS. For a separated experiment, kidney tissue sections from mice with Ang II pumps were incubated with polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) (500U/mL) at 37 °C for 30 min prior to 30-min exposure of DHE (20 µM). The tissue slides were wrapped with foil to minimize them exposure to light. Fluorescent images were observed with an Olympus Fluoview laser-scanning confocal microscope mounted on an Olympus microscope selected with CY3 (red) channel. One tissue slide was kept without DHE for blank control. Fluorescence was quantified by the ImageJ software (U.S. National Institutes of Health, Bethesda, MD).

Western Blot Analysis. Western blot analysis were carried out as previously described.⁶ Total protein was extracted from frozen kidney tissue by homogenization in EDTA-free RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP40, 0.1% SDS including protease inhibitor (Calbiochem, San Diego, USA) and phosphatase inhibitor cocktails (Sigma-Aldrich, Oakville, Canada) and quantified using the BCA Protein Array Kit (Pierce, Rockford, IL, USA). Protein samples were separated by 8%~12% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane (Millipore). The membrane was blocked with 5% milk in Tris-Buffered Saline Tween-20 (TBST) for 2 h and then incubated overnight at 4 °C with primary antibody against PKCα (80 kDa), PKCβ1 (79 kDa), ACE2 (90 kDa), Collagen I (150 kDa), Collagen III (70 kDa), β-actin (45 kDa) and total and phosphorylated ERK1 (44 kDa) and ERK2 (42 kDa) (Santa Cruz and Cell Signaling Inc.) as previously described.⁶ The primary antibodies against the AT1 receptor, Mas receptor and E-cadherin were obtained from Abcam (41kDa), Alomone labs, (~50kDa) and BD transduction laboratories (120kDa), respectively. After washing 3 times in TBST buffer, the membrane was then incubated with an secondary antibody at a 1:5000 dilution in TBST for 2 h at room temperature, then washed 3 times with TBST for 15 min each. Aim proteins were detected by enhanced chemiluminescence (GE) using X-O-Mat X-ray film (Fuji). The X-ray film was then scanned using a GS-800-calibrated densitometer

(Bio-Rad, Mississauga, Ontario, Canada) and band densities were measured using Quantity One software.

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SUPPLEMENTAL TABLES

Table S1. Primers and probes sequences for TaqMan real-time PCR analysis*.

Genes	Primers /Probes	Sequences (Primer: 5'-3'; Probe: 5'-FAM- -TAMRA-3')
<i>Pro-collagen I-α1</i>	Forward Primer	5'-CTTCACCTACAGCACCCCTTGTG-3'
	Reverse Primer	5'-TGACTGTCTTGCCCCAAGTTC-3'
	Probe	5'- FAM-CTGCACGAGTCACACC-TAMRA-3'
<i>Pro-collagen III-α1</i>	Forward Primer	5'-TGTCCTTTGCGATGACATAATCTG-3'
	Reverse Primer	5'-AATGGGATCTCTGGGTTGGG-3'
	Probe	5'-FAM-ATGAGGAGCCACTAGACT-TAMRA-3'
<i>TGFβ1</i>	Forward Primer	5'- CCTGCAAGACCATCGACATG-3'
	Reverse Primer	5'- ACAGGATCTGGCCACGGAT-3'
	Probe	5'- FAM-CTGGTGAAACGGAAGCGCATCGAA-TAMRA -3'
<i>TNFα</i>	Forward primer	5'- ACAAGGCTGCCCCGACTAC-3'
	Reverse primer	5'- TTTCTCCTGGTATGAGATAGCAAATC-3'
	Probe	5'-FAM-TGCTCCTCACCCACACCGTCAGC-TAMRA-3'
<i>IL-1β</i>	Forward primer	5'-AACCTGCTGGTGTGTGACGTTTC-3'
	Reverse primer	5'-CAGCACGAGGCTTTTTTGTGTG-3'
	Probe	5'- FAM-TTAGACAGCTGCACTACAGGCTCCGAGATG-TAMRA-3'
<i>IL-6</i>	Forward Primer	5'-ACAACCACGGCCTTCCCTACTT-3'
	Reverse Primer	5'-CACGATTTCCAGAGAACATGTG-3'
	Probe	5'-FAM-TTCACAGAGGATACCACTCCCAACAGACCT-TAMRA-3'

* Primer/probe mix for α -smooth muscle actin (α -SMA) (product #: Mm00725412_S1), NOX2 (product #: Mm01287743_m1), NOX 4 (product #: Mm01317083_m1), p47phox (product #: Mm00447920_g1), CCL5 (product #: Mm01302428_m1) and E-Cadherin (Mm01247357_m1) were purchased from Applied Biosystems Inc. TNF α , tumor necrosis factor α ; IL1 β , interleukin-1 β ; IL6, interleukin-6; TGF β 1, transforming growth factor- β 1.

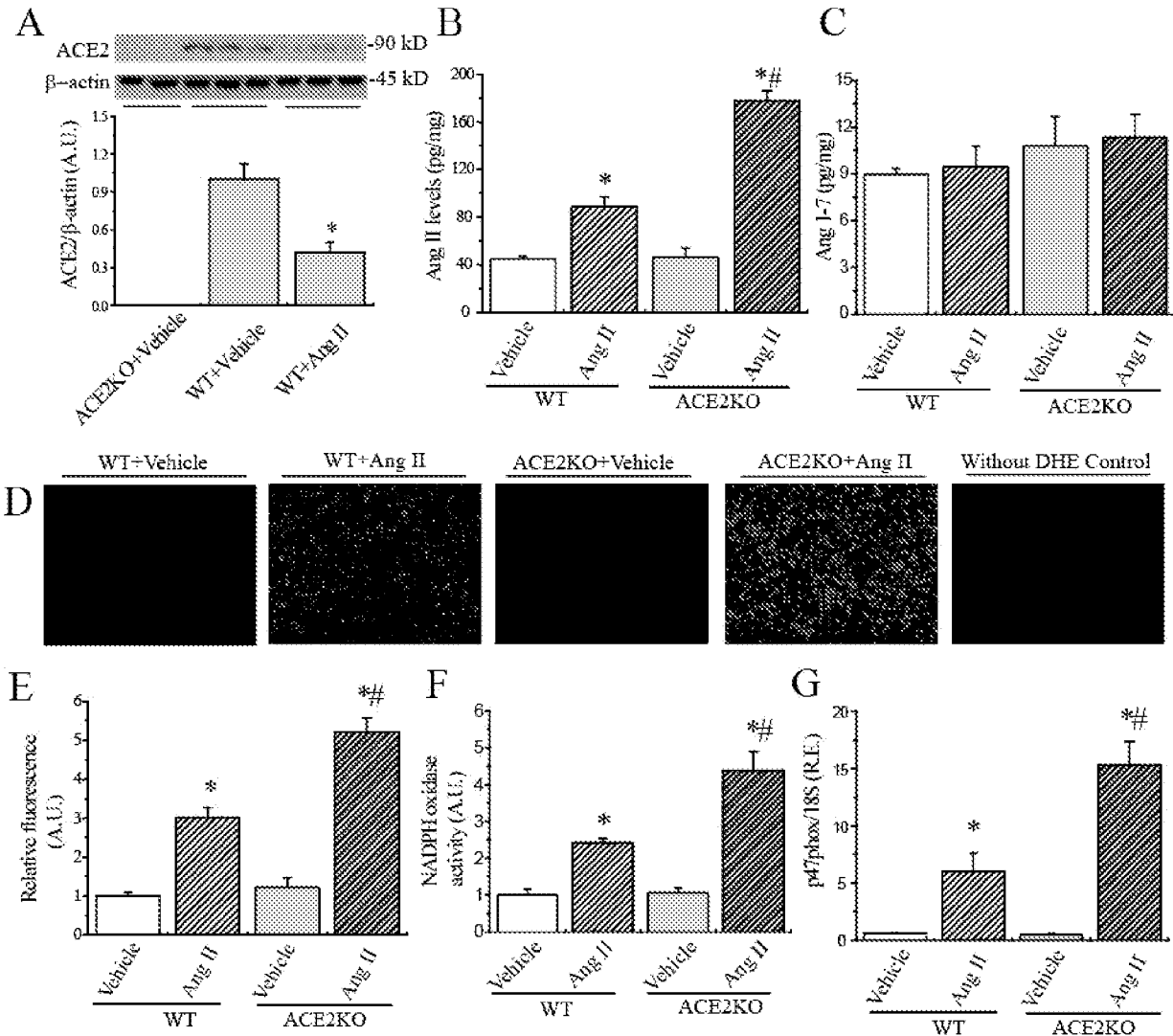


Figure S1. Loss of ACE2 increases Ang II levels, superoxide production and NADPH oxidase activity in response to Ang II. (A-B) Western blot analysis of renal ACE2 protein levels showing a marked reduction in response to Ang II (A) (n=4 for all groups; A.U.=Arbitrary Unit) and renal Ang II levels showing a greater elevation in ACE2KO kidneys compared with WT kidneys (B) without alteration in renal Ang 1-7 levels (C) in mice infused with Ang II (n=8 for vehicle-treated groups; n=14 for Ang II-treated groups). (D-E) Representative dihydroethidium fluorescence images (D), relative fluorescence values (E) and NADPH oxidase activity (F) showing greater superoxide generation and NADPH oxidase activity in ACE2KO mice in response to Ang II. (G-I) Taqman realtime PCR expression analysis showing greater renal expression of p47^{phox} (G) in response to Ang II in ACE2KO mice. NADPH, nicotinamide adenine dinucleotide phosphate. R.E.=Relative Expression; n=6 for WT+Vehicle, ACE2KO+Vehicle, n=8 for WT+Ang II, ACE2KO+Ang II. *p<0.05 compared with corresponding vehicle treated group; #p<0.05 compared with WT+Ang II group.

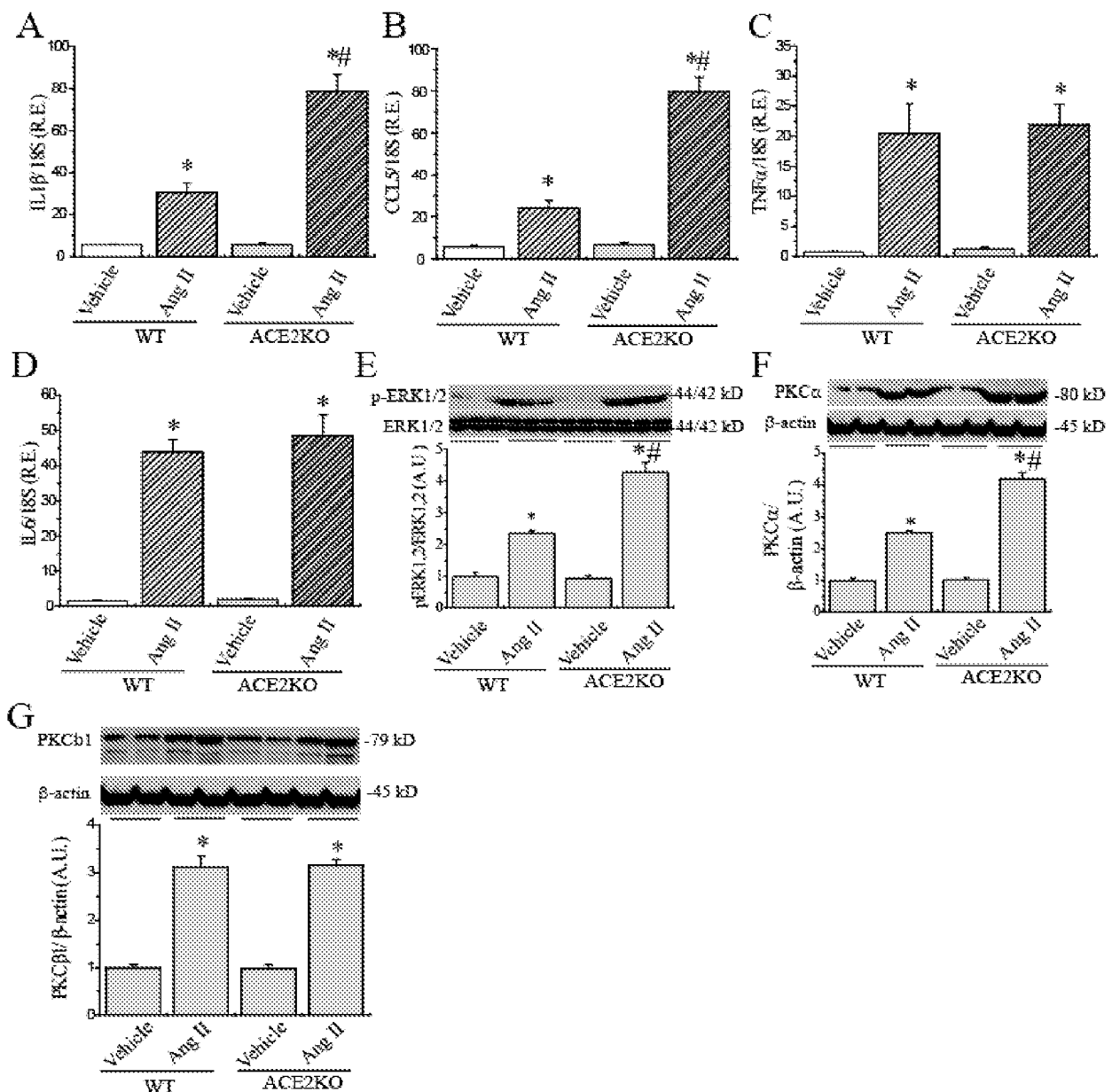


Figure S2. Loss of ACE2 increases the expression of inflammatory cytokines and activation of pathological signaling pathways in response to Ang II. (A-D) Taqman realtime PCR expression analysis of inflammatory showing greater elevation in IL1 β (A) and CCL5 (B) without a differential effect on TNF α (C) and IL6 (D) renal expression in response to Ang II in ACE2KO mice compared with WT mice. R.E.=Relative Expression; IL1 β =Interleukin-1 β ; TNF α =tumor necrosis factor alpha; IL6=Interleukin-6. n=6 for WT+Vehicle, ACE2KO+Vehicle, n=8 for WT+Ang II, ACE2KO+Ang II. (E-G) Western blot analysis in response to Ang II in ACE2KO mice compared with WT mice showing greater phosphorylation of ERK1/2 (E), increased PKC α levels (F) without a differential effect on PKC β 1 levels (G). n=4 for all groups. ERK1/2=Extracellular Regulated Kinase 1/2; PKC=protein kinase C. A.U.=Arbitrary Unit; *p<0.05 compared with corresponding vehicle treated group; #p<0.05 compared with WT+Ang II group.

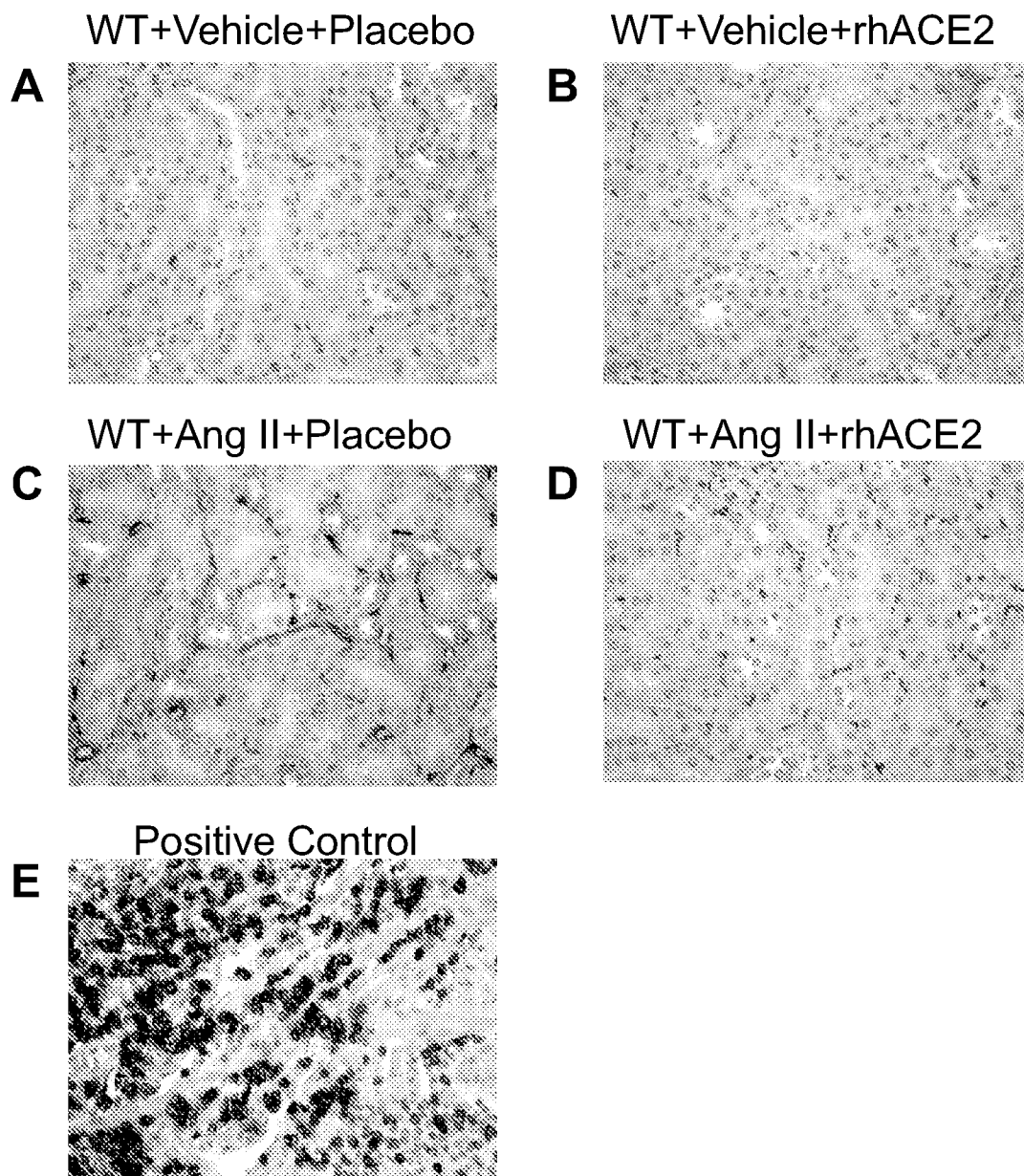


Figure S3. CD3-staining in paraffin-embedded kidney sections showing Ang II-induced infiltration of CD3 positive cells in the tubulointerstitium (x400 magnification) which was prevented by rhACE2. Positive control was obtained by staining tonsillar lymphoid tissue (E).

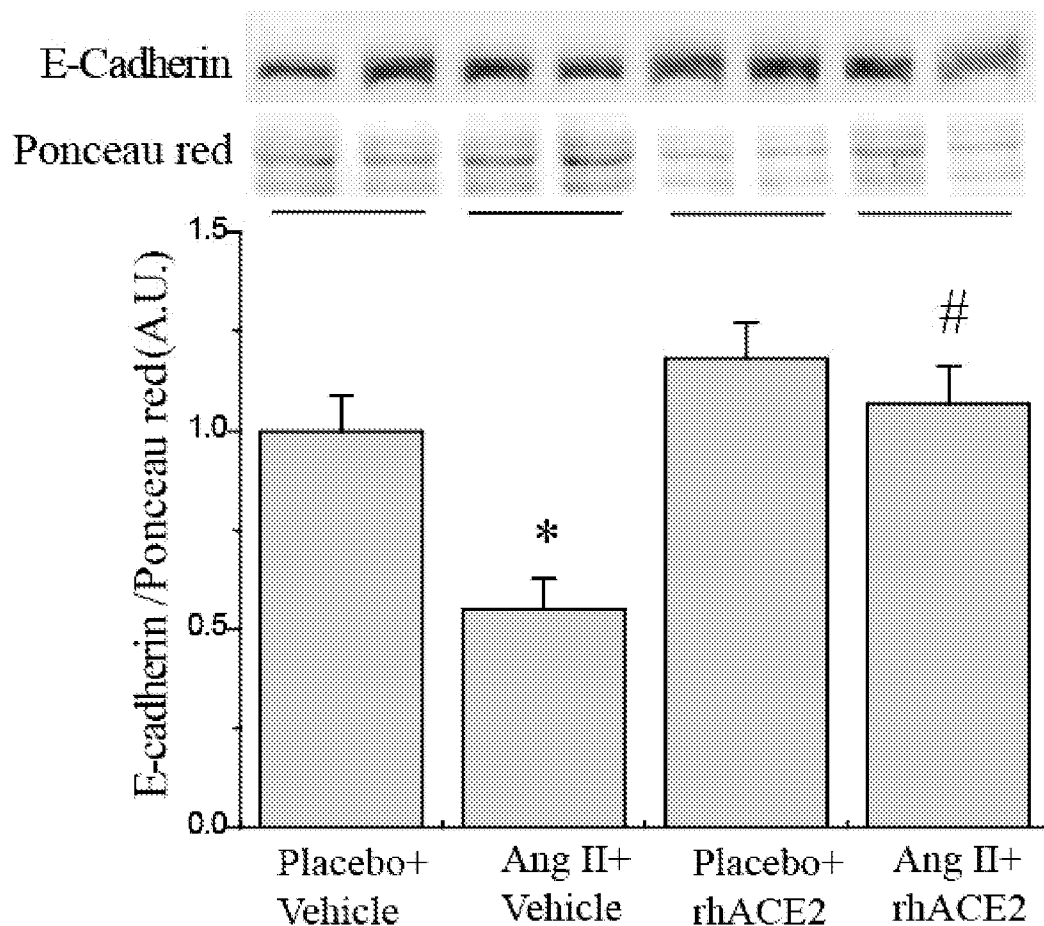


Figure S4. Western blot analysis of membrane fractionated E-cadherin in the kidneys showing that rhACE2 prevented the Ang II-mediated loss of E-cadherin. n=4 per group. * p<0.05 compared with all other groups; # p<0.05 compared with the WT+Placebo+Ang II group.